Use of Whole Genome Sequencing for the Molecular Comparison of *Neisseria gonorrhoeae* Isolates With Decreased Susceptibility to Extended Spectrum Cephalosporins From 2 Geographically Different Regions in America

Ricardo A. Gianecini, MSc, Sandra Zittermann, PhD,† Claudia Oviedo, MSc,* Marcelo Galas, BSc,‡ Pilar Ramon Pardo, MD,‡ Vanessa G. Allen, MD,† Patricia Galarza, MSc, MPH,* and Roberto G. Melano, PhD†*

Background: *Neisseria gonorrhoeae* isolates with reduced susceptibility or resistance to the recommended first-line antimicrobial therapy have been described in several countries. The purpose of this study was to use genome analyses to compare the molecular characteristics of *N. gonorrhoeae* isolates with decreased susceptibility to extended-spectrum cephalosporin from Ontario, Canada, and Argentina.

Methods: A total of 128 *N. gonorrhoeae* isolates, collected in 2015, were included. The susceptibility to penicillin G, tetracycline, ciprofloxacin, cefixime, ceftriaxone, and azithromycin was determined using the agar dilution method. Isolates were subjected to whole genome sequencing, and an in silico analysis was performed to identify antimicrobial resistance determinants and for genotyping.

Results: Decreased susceptibility to extended-spectrum cephalosporin was mainly associated with penA mosaic allele 34.001, together with an *mtrR* promoter A deletion and *porB1b* alterations G120K/A121N. *N. gonorrhoeae* multiantigen sequence typing ST1407 or closely related genotypes were identified circulating in both regions.

Conclusions: An international multi-drug resistant clone of *N. gonorrhoeae* was associated with decreased susceptibility to extended-spectrum cephalosporin (ESC) in 2 different regions in America. Evidence of clonal dissemination of the organism in some regions suggests that the strength of surveillance programs and establishment of collaborative projects are essential.

From the *National Reference Laboratory of Sexually Transmitted Diseases, National Institute of Infectious Diseases-ANLIS "Dr. Carlos G. Malbrán," Ciudad Autónoma de Buenos Aires, Argentina; †Public Health Ontario Laboratories, Toronto, Canada; and ‡Pan American Health Organization/ World Health Organization (PAHO/ WHO), Washington, DC

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Correspondence: Roberto G. Melano, Public Health Ontario Laboratories, Toronto, Canada. E-mail: roberto.melano@oahpp.ca.

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Since the introduction of antimicrobial therapy for gonorrhea, almost all antibiotic classes used for treatment have lost much of their efficacy owing to emergence of resistance.[1] In recent years, gonococcal clinical isolates with decreased susceptibility and resistance to the ESC ceftriaxone have been reported from many countries including Argentina and Canada.[1–5] These isolates have also resulted in treatment failures in several countries.[1,6] The emergence of ESC resistance has caused public health concern worldwide, and dual antimicrobial therapy (ceftriaxone plus azithromycin) is now recommended as first-line empirical treatment for uncomplicated gonorrhea in many countries.[7,8] Worryingly, the first case of treatment failure with dual therapy has recently been reported.[9] Moreover, a ceftriaxone-resistant strain described in Japan in 2015 (FC428) has spread internationally and been subsequently reported in several countries.[10] Recently, *Neisseria gonorrhoeae* isolates with ceftriaxone resistance and high-level azithromycin resistance have been identified in England and Australia.[11] This emergence threatens the effectiveness of the dual antimicrobial therapy.

Molecular and genetic epidemiologic studies have been used to describe *N. gonorrhoeae* isolates with decreased susceptibility and resistance to ESC in many settings worldwide.[12,13] These studies have identified gonococcal clones that are important for driving transmission of multi drug resistant gonorrhea within international networks.[12,13] Molecular epidemiological surveillance provides a measure of the stability and expansion of circulating strains, which together with an understanding of prevalence and distribution of clones may help in implementing appropriate measures for prevention and treatment. Therefore, molecular approaches including molecular epidemiological typing and detection of antimicrobial resistance determinants for gonococcal isolates with decreased susceptibility or resistance to ESC have become increasingly important.

The aim of this study was to analyze and compare the molecular characteristics of contemporary *N. gonorrhoeae* isolates with decreased susceptibility to ESC from Ontario, Canada, and Argentina.

MATERIALS AND METHODS

N. gonorrhoeae Isolates

A total of 128 clinical isolates of *N. gonorrhoeae* with decreased susceptibility to ceftriaxone (minimum inhibitory concentration [MIC] 0.06 to 0.25 μg/mL) and cefixime (MIC, 0.125 to

0.25 μg/mL) were selected for analysis: 57 from the National Institute of Infectious Diseases-ANLIS "Dr. Carlos G. Malbrán," Argentina (all the isolates with this phenotype collected in 2015), and 71 from Public Health Ontario (an equivalent and representative number of *N. gonorrhoeae* isolates collected in Ontario, Canada, during the same period). The isolates from Ontario were obtained from Toronto (72.6%), Ottawa (12.3%), Hamilton (5.5%), London (8.2%), and Sudbury (1.4%); these 71 isolates represent approximately 10% of the total number of isolates for the province in 2015. Meanwhile, the isolates from Argentina were identified in Buenos Aires (19.4%), Córdoba (61.4%), Neuquén (7.0%), Santa Fe (5.3%), Rio Negro (3.5%), Mendoza (1.7%), and Chaco (1.7%). These isolates represented 7.8% of the total isolates in Argentina in the period of study. Identification of the *N. gonorrhoeae* isolates was confirmed by culture on selective agar media, Gram staining, oxidase test, superoxol test (30% hydrogen peroxide), carbohydrate utilization test, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Microflex LT, Bruker Daltonik, Bremen, Germany).[14] Isolates were preserved at −80°C in trypticase soy broth containing 20% glycerol.

Antimicrobial Susceptibility Testing

All isolates were subcultured on Difco GC medium base agar (BD, Franklin Lakes, NJ, USA) supplemented with 1% enrichment supplement for 18 to 24 hours at 35°C in a humid 5% CO_2-enriched atmosphere before testing. The resistance profiles of *N. gonorrhoeae* isolates to cefixime (Bagó Laboratories, Buenos Aires, Argentina; USP Reference Standard, Canada), penicillin, tetracycline, ceftriaxone, erythromycin (Richet Laboratories, Buenos Aires, Argentina; Sigma, Canada); ciprofloxacin and azithromycin (Richet Laboratories, Buenos Aires, Argentina; USP Reference Standard, Canada) were determined using the agar dilution method, and CLSI guidelines were used to interpret the results except for azithromycin, where the US Centers for Disease Control and Prevention guidelines were used ($R \geq 2$ μg/mL).[15,16] In order to identify isolates with decreased susceptibility to azithromycin, the epidemiological cutoff value of 1 μg/mL established by European Committee on Antimicrobial Susceptibility Testing was used.[17] *N. gonorrhoeae* ATCC 49226 and the 2008 WHO *N. gonorrhoeae* reference strains panel were used as quality control strains.[18]

In this study, multidrug resistant *N. gonorrhoeae* (MDR-NG) was defined as "decreased susceptibility/resistance to 1 currently recommended therapy (cephalosporin: ceftriaxone MIC ≥0.125 μg/mL; cefixime MIC ≥0.25 μg/mL or azithromycin) plus resistance to at least 2 other antimicrobials (penicillin, tetracycline, erythromycin, ciprofloxacin)."[19]

DNA Sequencing

Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions, and eluted in 100 μL of AE buffer. DNA concentration was determined by Qubit® 2.0 Fluorometer (Thermo-Fisher Scientific), and DNA samples were stored at −20°C until further processing. The sequencing library was prepared using the Illumina Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA) as per manufacturer's instructions. Agilent 2100 Bioanalyzer was used to determine quality of DNA library. Sequencing was performed at the Centre for the Analysis of Genome Evolution and Function (CAGEF, University of Toronto) using the NextSeq500 Desktop Sequencer v2 or at Public Health Ontario Laboratory with an Illumina MiSeq platform with 600-cycle MiSeq Reagent Kit v3.

Sequence Assembly and Detection of Resistance Genes

The quality of the reads was assessed using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). De novo assembly was performed using Spades v.3.9.0 (http://spades.bioinf.spbau.ru/release3.9.0/manual.html) with the command line options "-k 33, 55, 77, 99, 127" and "—careful." For analysis of resistance determinants, in silico PCRs performed using a custom Perl script were conducted to extract *penA*, the full length mtr locus (*mtrR*, its promoter and the *mtrCDE* efflux pump operon), *porB*, *ponA*, *gyrA*, *parC*, *rplV*, and *rplD* from the de novo assembly. The Perl script blast searched for short nucleotide sequences with homology to the beginning and end of each gene in the genome sequences. If the pair of short sequences was found, the size of the sequence was determined and if matching the predicted size, the sequence was included in the analysis. We used *N. gonorrhoeae* sequence typing for antimicrobial resistance molecular typing scheme to identify mutations in penA and ponA genes.[20] The *mtr* locus, *porB*, *gyrA*, *parC*, *rplV*, and *rplD* sequences were aligned and compared with reference sequences using Mega software version 7. The GenBank accession numbers of the reference sequences used were as follows: Z25796 (*mtrR*), J03017 (*porB1b*), and AE004969.1 (*gyrA*, *parC*, *rplV*, and *rplD*).

To assess macrolide resistance, we examined mutations in the peptidyltransferase region of domain V of the 23S rRNA gene and the *mtr* locus. A reference sequence was created by concatenating 1 wild-type copy of the 23S rRNA gene and 1 copy of the 23S rRNA gene with substitutions at positions A2059G and C2611T.[21] For each strain, sequence reads were mapped to this reference sequence using SMALT software version 0.7.6 (http://www.sanger.ac.uk/science/tools/smalt-0). The output was sorted and indexed using SAMtools v1.7 (http://www.htslib.org/doc/samtools.html) to generate a BAM file. The mapped reads were visualized using Tablet software (https://ics.hutton.ac.uk/tablet/). The number of mutant copies of the 23S rRNA gene was determined by dividing the number of reads mapping to A2059G or C2611T position by the total number of reads mapped to the reference sequence. Previous studies have shown that gonococci have 4 copies of 23S rRNA and it is possible to harbor mixture of both wild-type and mutant alleles between these 4 copies.[21] Therefore, the rates were rounded to the nearest quartile.

Phylogenetic Analysis

N. gonorrhoeae multiantigen sequence typing (NG-MAST) gene sequences were identified in silico from WGS data.[22] Sequences were trimmed, using Mega version 7.0, to define start and end positions according to NG-MAST, and to yield fragments of the following sizes: 490 for *porB* and 390 for *tbpB*. The sequences were submitted to the NG-MAST website (http://www.ng-mast.net/) to determine the sequence types (STs). Closely related STs were clustered using previously described genogroup definition.[13] Accordingly, STs that shared 1 identical allele with the other allele showing 99% or greater identity were clustered together.

The trimmed sequences were concatenated in the order *porB-tbpB* to generate an 880-pb sequence, and a maximum parsimony tree was generated using Bionumerics 6.6 (Applied Maths). Single-nucleotide variant (SNV) analysis was conducted using a custom pipeline. Briefly, reads for all isolates were mapped against the chromosome of the NCCP11945 reference strain (GenBank accession number NC_011035) using SMALT software (v 0.7.6). Single-nucleotide polymorphism calling was

TABLE 1. Antimicrobial Susceptibility Profiles of 95 *N. gonorrhoeae* Isolates Exhibiting Decreased Susceptibility to Ceftriaxone and Cefixime

	Argentinean Isolates (n = 24)				Canadian Isolates (n = 71)			
Antimicrobial	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	MIC Range (μg/mL)	% Resistant Isolates	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	MIC Range (μg/mL)	% Resistant Isolates
Penicillin	2	4	1–4	87.5	1	2	0.125 to ≥4	16.9
Tetracycline	4	4	0.25–4	95.8	2	4	1 to ≥64	64.8
Ciprofloxacin	16	16	4–16	100	≥1	≥1	≤0.03 to ≥1	98.6
Ceftriaxone	0.06	0.125	0.06–0.25	0	0.06	0.125	0.125–0.25	0
Cefixime	0.25	0.25	0.125–0.25	0	0.125	0.25	≤0.03–0.25	0
Azithromycin	0.5	1	0.03–1	0	≤0.25	0.5	≤0.25 to ≥4	4.2

MIC, minimum inhibitory concentration.

performed using Freebayes with – min-base-quality 30, – min-mapping-quality 30, – min-alternate-fraction 0.75, – read-snp-limit 10, – min-coverage 15. Additional variant confirmation was done using the SAMtools mpileup tool. Repetitive regions were removed by using MUMmer. The meta-alignment of core informative positions was used to create a maximum likelihood tree using MEGA 7.

RESULTS

From the 57 *N. gonorrhoeae* isolates from Argentina included in this study, 35 were determined to be clonal (NG-MAST ST1407 or closely related), and epidemiological data confirmed that they belonged to an outbreak in the Córdoba province (Supplementary Fig. 1, http://links.lww.com/OLQ/A383). For that reason, they were excluded from this study with the exception of 2 of these isolates, which were kept in the analysis as representative of the outbreak. Therefore, a total of 95 *N. gonorrhoeae* isolates (n = 71) from Canada and (n = 24) from Argentina were included in the analysis. Most of the isolates (82.1%, n = 78) were collected from men, 14.7% (n = 14) were from female and the sex was unknown for the remaining 3 isolates. The age range of the patients was younger than 1 year to 69 years and 52.6% were younger than 29 years. The site of samples was mainly genital (75.7%), followed by pharyngeal (9.5%), rectal (7.4%), and others (7.4%).

Antimicrobial Resistance

The susceptibility profiles of 95 *N. gonorrhoeae* isolates exhibiting decreased susceptibility to ceftriaxone and cefixime are presented in Table 1. Resistance to ciprofloxacin was observed in 100% of Argentinean isolates and 98.6% of Ontarian isolates. Penicillin and tetracycline resistance rates were higher in Argentinean than in Ontarian isolates (penicillin, 87.5% vs. 16.9%; tetracycline, 95.8% vs. 64.8%). However, 59 (83.1%) and 25 (35.2%) isolates from Ontario displayed intermediate resistance to penicillin (MIC, 0.125 to 1 μg/mL) and tetracycline (MIC, 1 μg/mL), respectively. Resistance to azithromycin was observed only in 3 (4.2%) isolates from Ontario (MIC ≥4 μg/mL). However, 11 (45.8%) isolates from Argentina and 1 (1.4%) from Ontario showed decreased azithromycin susceptibility (MIC, 1 μg/mL) and were above the azithromycin European Committee on Antimicrobial Susceptibility Testing epidemiological cutoff.[17] Of the 95 isolates studied, 33.7% (n = 32) were MDR-NG. The proportion of MDR-NG in Argentinean isolates was 62.5% (15/24), compared with 23.9% (17/71) in Ontarian isolates.

Molecular Characterization

The presence of mutations associated with β-lactam (*penA*, *mtrR*, *porB*, and *ponA*), ciprofloxacin (*gyrA* and *parC*) and azithromycin (*rplV*, *rplD* and 23S rRNA) resistance are shown in Table 2 (mtr locus analysis for azithromycin resistance is described later). All the isolates (except one from Ontario, wild-type for both parC and gyrA genes) were gyrA/parC double mutants, displaying high MICs for ciprofloxacin (MIC 4–32 μg/mL). Among all 95 isolates investigated, 86.3% (n = 82) contained a mosaic *penA* allele. Fifty-one (53.7%) and 31 (32.6%) of the isolates contained the *penA* mosaic allele 34.001 and 10.001, respectively. The *penA* mosaic 34.001 was predominant in Argentinean (75%, 18 of 24) and Ontarian (46.5%, 33 of 71) isolates. In addition, the *penA* mosaic 10.001 was also commonly observed in isolates from Ontario (42.2%; 30 of 71), with only 1 isolate from Argentina carrying that allele. The remaining 13 (13.7%) isolates showed non-mosaic alleles: *penA* 13.001 and 44.001 were detected in isolates from Ontario (8.4%, 6 of 71; and 1.4%, 1 of 71, respectively) and Argentina (8.3%, 2 of 24; and 4.2%, 1 of 24, respectively), whereas alleles 5.002 and 43.002 were only observed in 2 isolates from Argentina (8.3%) and 1 from Ontario (1.4%), respectively (Table 2). These non-mosaic *penA* alleles encode for PBP2 with specific amino acid substitutions (*penA*-13.001, A501V and P551S; *penA*-44.001, A501T and P551L; *penA*-5.002, G542S; and *penA*-43.002, A501V), previously associated with decreased susceptibility to ESCs.[23] Also, all isolates carrying non-mosaic *penA* genes additionally contained mutations in *mtrR* and *porB1b* genes, suggesting that the combination of mechanisms have contributed to decreased susceptibility to ESC in these isolates.

Mutations in the promoter region or coding sequence of *mtrR* gene were observed in all the isolates. A single-adenine deletion in the promoter region was observed in 66.3% (63 of 95) of them, but amino acid changes in the predicted sequence of MtrR were found in all isolates as follows: 53 (55.8%) contained H105Y (always in combination with an adenine deletion in the promoter region), 29 (30.5%) contained A40D/T86A, 7 (7.4%) contained G45D, and 2 (2.1%) contained D79N/T86A/H105Y (these last 2 patterns of substitution also with an adenine deletion in the promoter region). Three isolates showed substitutions N34T/H105Y, A39T/R44H and A39T/F62L, and 1 isolate had a single-cytosine deletion at the MtrR binding site (mtr_{-72}) in the *mtrCDE* promoter and A39T in the predicted amino acid sequence of MtrR.

Missense mutations in *porB1b* were observed in 95.8% (91 of 95) of the isolates, suggesting a decreased intake of antimicrobials. Isolates had *porB1b* mutations that resulted in amino acid substitutions at positions 120 (G120K, N) and 121 (A121N, D, G, S). Substitutions G120K/A121N were observed in 51.6% (49 of 95) of the isolates, followed by G120K/A121D in 40% of the isolates (38 of 95). Three isolates were A121S/N122K and 1 was G120N/A121G. Substitution L421P in PBP1 was observed in 98.9% (94 of 95) of the isolates.

Two to 4 cumulative mutations in the QRDR of *gyrA* and *parC* genes were detected in all isolates with resistance to ciprofloxacin. All but 1 isolate had double GyrA substitutions in

TABLE 2. Mutation Patterns in ARGENTINEAN and CANADIAN *N. gonorrhoeae* Isolates With Decreased Susceptibility to ESC

		Genetic Features										
		β-Lactams				Azithromycin			Ciprofloxacin			
Strains, *n*		PBP2 allele*,†	PorB‡	PBP1	MtrR§,¶	L22 Protein	L4 Protein	23s rRNA 2059/2611 (No. Mutated Alleles)	GyrA	ParC	NG-MAST STs	NG-STAR Types
Argentinean strains	18	34.001*	G120K;A121N	L421P	A-§;H105Y	WT	WT	WT/WT	S91F;D95G	S87R	1407, 2212, 3149, 3294, 5791, 6314, 15461, 16030, 16031	90
	2	13.001†	G120K; A121D	L421P	A-;G45D	WT	WT	WT/WT	S91F;D95G	D86N	2318	38
	2	5.002†	NA	L421P	A-;H105Y	WT	WT	WT/WT	S91F;D95G	S87R	8509, 16032	937
	1	10.001*	G120K; A121D	L421P	A-;H105Y	WT	WT	WT/WT	S91F;D95G	S87R	2958	22
	1	44.001†	WT	L421P	A-;D79N;T86A; H105Y	WT	WT	WT/WT	S91F;D95G	E91G	16034	419
Canadian strains	23	34.001*	G120K;A121N	L421P	A-;H105Y	WT	WT	WT/WT	S91F;D95G	S87R	1407, 10451, 4822	90
	3	34.001*	G120K;A121N	L421P	A-;H105Y	WT	WT	WT/C2611T (4)	S91F;D95G	S87R	10451	91
	3	34.001*	G120K;A121N	L421P	A-;H105Y	WT	WT	WT/C2611T (1 or 2)	S91F;D95G	S87R	10451	90
	1	34.001*	G120K;A121N	L421P	A-;H105Y	WT	WT	WT/WT	S91F;D95G	G85C;S87R	10451	90
	1	34.001*	A121S;N122K	L421P	A-;H105Y	WT	WT	WT/WT	S91F;D95G	S87R	13902	250
	1	34.001*	G120K;A121N	L421P	A-;N34 T;H105Y	G26C	WT	WT/WT	S91F;D95G	E91G	10451	1480
	1	34.001*	WT	WT	A39T;R44H	WT	V125A;A147G; R157Q	WT/WT	WT	WT	2992	454
	26	10.001*	G120K;A121D	L421P	A40D;T86A	WT	WT	WT/WT	S91F;D95N	S87R;S88P	5308, 7554, 8171,17080	348
	1	10.001*	G120K;A121D	L421P	A40D;T86A	WT	WT	WT/WT	S91F;D95G	S87R;S88P	8171	348
	1	10.001*	G120K;A121D	L421P	A40D;T86A	WT	I126S	WT/WT	S91F;D95G	I52S;E91G	7554	1479
	1	10.001*	G120 N;A121G	L421P	A40D;T86A	WT	WT	WT/WT	S91F;D95N	S87R	10557	348
	1	10.001*	G120K;A121D	L421P	A39T;F62 L	WT	WT	WT/WT	S91F;D95N	G85C	12428	490
	4	13.001†	G120K;A121D	L421P	A-;G45D	WT	WT	WT/WT	S91F;D95G	D86N	2318	38
	1	13.001†	G120K;A121D	L421P	C-72¶;A39T	WT	WT	WT/WT	S91F;D95G	D86N	2318	38
	1	13.001†	A121S;N122K	L421P	A-;G45D	WT	WT	WT/WT	S91F;D95G	D86N	13512	38
	1	43.002†	G120K;A121D	L421P	A-;H105Y	WT	WT	WT/WT	S91F;D95A	S87R	7741	345
	1	44.001†	A121S;N122K	L421P	A-;D79N;T86A; H105Y	WT	WT	WT/WT	S91F;D95G	E91G	9663	480

*Mosaic PBP2 allele.
†Non-mosaic PBP2 allele.
‡NA for *porB* gene indicates the presence of a *porB1a* allele.
§A-, adenine deletion in the promoter region.
¶C-deletion at position −72 of *mtrR*.
NG-MAST, *N. gonorrhoeae* multiantigen sequence typing; NG-STAR, *N. gonorrhoeae* sequence typing for antimicrobial resistance; ST, sequence type.
NG-STAR, *N. gonorrhoeae* sequence typing for antimicrobial resistance.

positions S91F and D95G, D95N or D95A combined with ParC substitutions in positions G85C, D86N, S87R or E91G. Additional parC substitutions in some isolates accompanied the changes mentioned above (I52S, S88P, G85C) (Table 2). Three azithromycin-resistant isolates (MIC ≥4 μg/mL) contained the previously described C2611T mutation in all 4 alleles of the 23S rRNA gene. Among the 12 isolates with decreased susceptibility to azithromycin (MIC, 1 μg/mL), one had the C2611T mutation in 2 alleles, but the others were wild type for all 4 23S rRNA genes. All these isolates contained a single-adenine deletion in the *mtrR* promoter and the H105Y mutation in the MtrR. Recently, it has been shown that the gonococcal mtr locus is a hot spot for interspecies recombination and that *N. gonorrhoeae* isolates with mosaic *mtr* loci (i.e., *N gonorrhoeae* isolates with *N. lactamica* or *N. meningitidis*-like *mtr* loci), are "less-azithromycin-susceptible" (MIC values of 1–4 μg/mL).[24,25] We found 2 isolates from Ontario with a mosaic *mtr* locus, 1 with azithromycin MIC 1 μg/mL and 1 with MIC of 0.25 μg/mL or less. The identity between these 2 isolate's *mtr* loci was 95%, with the highest number of differences in the *mtr* promoter region (data not shown). The NG-MAST types were different. None of the isolates from Argentina with azithromycin MIC of 1 μg/mL had mosaic mtr loci. Furthermore, none of these isolates possessed chromosomal mutations in the *rplV* and *rplD* genes. Isolates with mutations in L4 or L22 proteins had MICs to azithromycin of 0.25 μg/mL or less and 0.5 μg/mL.

NG-MAST and Phylogenetic Analysis

Based on NG-MAST analysis, 14 STs were identified in the 24 strains from Argentina, 4 of which were new (ST16030,

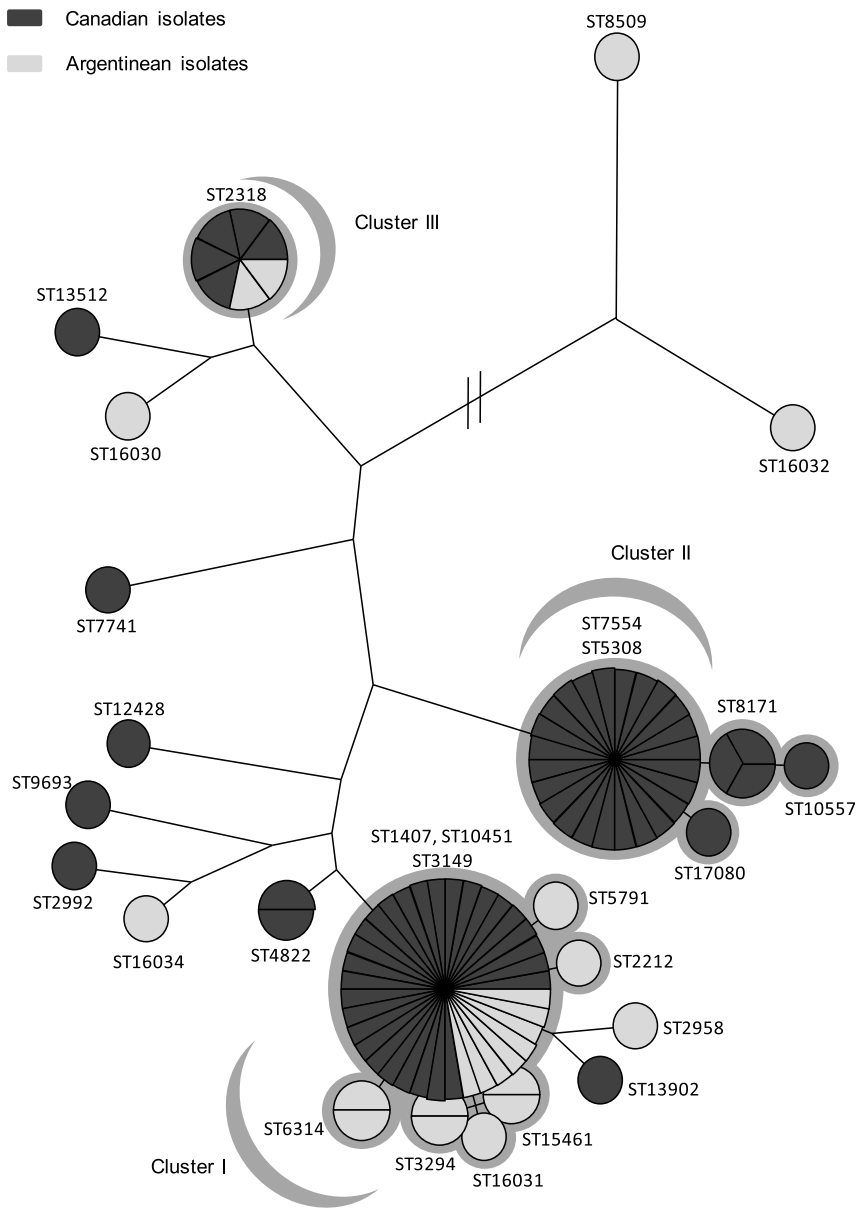

Figure 1. Maximum parsimony tree based on concatenated sequences of *porB* and *tbpB* genes describing distribution of *N. gonorrhoeae* isolates from Argentina and Canada. Each cluster is represented by a gray halo surrounding circle.

TABLE 3. Different STs Within the 3 Clusters Defined Based on Sequence Similarity at *porB* and *tbpB* Allele

Genogroup, n (%)*	ST (n)	Allele *porB*	*tbpB*	Argentinean Isolates, n (%)†	Canadian Isolates, n (%)‡
Cluster I 46 (48.4)	10,451 (27)	6106	110	0 (0)	27 (38)
	1407 (8)	908	110	6 (25)	2 (2.8)
	2212 (1)	1388	110	1 (4.2)	0 (0)
	3149 (2)	1903	110	2 (8.3)	0 (0)
	3294 (2)	1994	110	2 (8.3)	0 (0)
	5791 (1)	3468	110	1 (4.2)	0 (0)
	6314 (2)	3725	110	2 (8.3)	0 (0)
	15461 (2)	9043	110	2 (8.3)	0 (0)
	16031 (1)	9353	110	1 (4.2)	0 (0)
Cluster II 29 (30.5)	7554 (23)	4539	10	0 (0)	23 (32.4)
	8171 (3)	4901	10	0 (0)	3 (4.2)
	10,557 (1)	6171	10	0 (0)	1 (1.4)
	5308 (1)	3215	10	0 (0)	1 (1.4)
	17,080 (1)	9958	10	0 (0)	1 (1.4)
Cluster III 7 (7.4)	2318 (7)	1053	4	2 (8.3)	5 (7.0)

*A total of 95 isolates from Argentina and Ontario, Canada, were studied.
†A total of 24 isolates were studied from Argentina.
‡A total of 71 isolates were studied from Ontario, Canada.
ST, sequence type.

ST16031, ST16032, and ST16034). The isolates from Ontario belonged to 15 distinct STs. Only ST1407 and ST2318 were found in both places. A phylogenetic tree was generated using concatenated sequences of the porB (490 pb) and tbpB (390 pb) genes. A cluster was defined as a group of STs which shared 1 allele and showed 99% or greater similarity in the other allele (≤5 bp difference for *porB* and ≤4 bp for *tbpB*) or an ST with 2 different alleles, but the concatenated sequence of both alleles (880 bp) displayed 99% or greater similarity to the concatenated sequence of both alleles of the main ST in the cluster. Eighty-two isolates were grouped into 3 clusters (Fig. 1). Cluster I (n = 46) was the major cluster containing 17 (70.8%) isolates from Argentina and 29 (40.8%) from Ontario. The isolates were from male (86.9%) and female patients (10.9%). This cluster consisted of 9 different STs, being ST1407 (*tbpB* allele 110 and *porB* allele 908) common in both regions. The remaining STs in this cluster showed tbpB allele 110 and 99% or greater similarity with porB allele 908, indicating that these STs were closely related to ST1407 (Table 3). All isolates in Cluster I were resistant to ciprofloxacin, while the resistance rates to penicillin and tetracycline were higher in isolates from Argentina than in those from Ontario (penicillin, 94.1% vs. 24.1%; tetracycline, 100% vs. 96.5%). Three (10.3%) of the isolates from Ontario were resistant to azithromycin, but 10 (58.8%) isolates from Argentina and 1 (3.4%) from Ontario showed decreased susceptibility to azithromycin. Cluster II grouped 29 isolates only from Ontario and was mainly associated with ST7554 or closely related STs. The isolates were cultured from males (72.4%) and females (24.1%), and were

TABLE 4. Distribution of Isolates According to Cefixime and Ceftriaxone MIC and Mutations in *penA, porB,* and *ponA* Genes and *mtrR* Promoter and Gene

Cefixime, μg/mL (No. Isolates/Total)	Ceftriaxone, μg/mL (No. Isolates/Total)*	*penA* allele†	*PorB*	*mtrR* Prom/MtrR‡	PBP1	No. Isolates (%)
0.25 (45/95)	≤0.06 (31/45)	10.001	G120K, A121D	WT/A40D, T86A	L421P	18/31 (58)
		34.001	G120K, A121N	−35A Del/H105Y	L421P	12/31 (39)
		5.002	N/A	−35A Del/H105Y	L421P	1/31 (3)
	0.12 (13/45)	10.001	G120K, A121D	WT/A40D, T86A	L421P	10/13 (77)
		34.001	G120K, A121N	−35A Del/H105Y	L421P	1/13 (8)
		44.001	A121S, N122K	−35A Del/D79N, T86A, H105Y	L421P	1/13 (8)
		13.001	G120K, A121D	−72C Del/A39T	L421P	1/13 (8)
	0.25 (1/45)	10.001	G120K, A121D	WT/A40D, T86A	L421P	1/1 (100)
0.125 (50/95)	≤0.06 (44/50)	10.001	G120K, A121D	WT/A40D, T86A	L421P	2/44 (5)
		34.001	G120K, A121N	−35A Del/H105Y	L421P	38/44 (86)
		44.001	WT	−35A Del/D79N, T86A, H105Y	L421P	1/44 (2)
		13.001	G120K, A121D	−35A Del/H105Y	L421P	2/44 (5)
		43.002	G120K, A121D	−35A Del/H105Y	L421P	1/44 (2)
	0.12 (5/50)	13.001	G120K, A121D	−35A Del/H105Y	L421P	5/5 (100)
	0.25 (1/50)	5.002	N/A	−35A Del/H105Y	L421P	1/1 (100)

*Twenty-eight Canadian isolates had cefixime MICs of 0.125 to 0.25 μg/mL but ceftriaxone MIC ≤0.03 μg/mL. All the Argentinian isolates had ceftriaxone MIC = 0.06 μg/mL.
†According to NG-STAR. Total number of isolates with each penA allele are: 10.001, n = 31; 34.001, n = 51; 5.002, n = 2; 44.001, n = 2; 13.001, n = 8; 43.002, n = 1.
‡Mutations in the promoter of *mtrR* gene ('Del', deletion), or amino acid changes in the MtrR repressor.

resistant to penicillin (3.4%), tetracycline (31%), and ciprofloxacin (100%). Cluster III (n = 7) was composed of ST2318 and contained 2 isolates from Argentina (8.3%) and 5 from Ontario (7.0%). Six of the isolates were isolated from male patients. All isolates in cluster III displayed resistance to ciprofloxacin (100%) and tetracycline (100%). However, resistance to penicillin was observed in 40% of Ontarian isolates and 100% of Argentinean isolates. None of the isolates in cluster III showed decreased susceptibility to azithromycin. Although the NG-MAST typing scheme has been proven useful to establish *N. gonorrhoeae* phylogenetic relationship, SNV analysis provides a higher discriminatory power.[13] In our study, a core genome SNV analysis of the isolates (including the 35 gonococci from an outbreak in Córdoba province) defined the same 3 clusters, in concordance with the NG-MAST analysis (Supplementary Fig. 1, http://links.lww.com/OLQ/A383).

Mutations in the *penA, mtrR, porB* and *ponA* genes have been reported to influence cephalosporin susceptibility[23] (Table 4). We observed that 94% of isolates with *penA* mosaic 10.001 (29 of 31) had cefixime MICs of 0.25 μg/mL, whereas only 25% of the isolates with *penA* mosaic 34.001 (13 of 51) displayed that MIC. In contrast, 75% of the isolates with a penA 34.001 and 6% of the isolates with *penA* 10.001 had cefixime MICs of 0.125 μg/mL. Furthermore, 79% of isolates with cefixime MIC 0.25 μg/mL and ceftriaxone of 0.125 μg/mL or greater had a *penA* 10.001. These different mutation patterns were significantly associated with each group. Isolates from Cluster I (45 of 46, 97.8%) predominantly had *penA* mosaic 34.001, an A-deletion in *mtrR* promoter, position −35, together with MtrR H105Y amino acid change, porin G120K/A121N changes, and L421P substitution in PBP1. Cluster II (28 of 29, 96.5%) was associated with *penA* mosaic 10.001, MtrR A40D/T86A amino acid changes, porin G120K/A121D changes, and L421P substitution in PBP1, whereas cluster III (6 of 7, 85.7%) was associated with *penA* non-mosaic 13.001, an A-deletion in *mtrR* promoter at position −35 together with MtrR G45D amino acid change, porin G120K/A121D changes and L421P substitution in PBP1.

DISCUSSION

This study provides novel information for phenotypic and molecular data regarding drug resistance in *N. gonorrhoeae* isolates from 2 geographically different regions in America. A comparison of genotypic characteristics of *N. gonorrhoeae* strains with decreased susceptibility to ESC from Argentina and Ontario, Canada, identified a clonal group circulating in both regions.

Isolates with decreased susceptibility to ESC in Argentina showed also a general, higher level of resistance compared to the isolates in Ontario, with most of them (62.5%) showing a MDR-NG profile. These results indicate that MDR phenotypes are still present in Argentina since detected in 2011.[2] In Argentina, national guidelines recommend monotherapy with ceftriaxone (125–250 mg IM in a single dose) or cefixime (400 mg orally in a single dose) as first-line treatment for uncomplicated gonorrhea.[26,27] Although macrolides and tetracyclines are not used for its treatment, azithromycin (1 g orally in a single dose) or doxycycline (100 mg orally twice a day for 7 days) are recommended for treatment of *Chlamydia trachomatis* or *Mycoplasma genitalium* infections.[26,27] In addition, Argentinian guidelines recommend antimicrobial treatment if the infection is suspected but not confirmed. This practice may represent a risk of selecting resistant *N. gonorrhoeae* isolates. Dual antimicrobial therapy with ceftriaxone plus azithromycin is now recommended as first-line treatment in many regions, including Ontario, as a strategy to stem the development of antimicrobial resistance in *N. gonorrhoeae*.[7,8,28] Consequently, dual antimicrobial therapy (e.g., ceftriaxone 250/500 mg plus azithromycin 1 g) might need to be considered in Argentina for all gonorrhea cases to mitigate the selection of MDR-NG isolates.

In both regions, the isolates with decreased susceptibility to ESC showed a high percentage of ciprofloxacin resistance, and cumulative mutations were observed in the QRDR *gyrA* and *parC*.[23] *N. gonorrhoeae* isolates from Ontario showed high genetic variability, and a significant proportion were associated with intermediate resistance to penicillin (83.1%) and tetracycline (35.2%) as previously described.[12] In our study, decreased susceptibility to ESC was mainly associated with penA mosaic allele 34.001, which is commonly found in NG-MAST ST1407 and related clones.[13] That is the case of F89, the second clinical isolate characterized as XDR, which displayed high level resistance to ceftriaxone (MIC 2 μg/mL) and cefixime (MIC 4 μg/mL) due to an additional A501P amino acid substitution in its PBP2 (penA 34.001).[23] The presence of ST1407 and related STs increases the risk of ESC resistance emergence in the region. *penA* mosaic 10.001 was more frequently found in Ontarian than in Argentinean isolates. This *penA* type was associated in our samples with higher MIC to both cefixime and ceftriaxone, although they were susceptible to azithromycin. The emergence of azithromycin resistance is also of concern, as it is recommended with ceftriaxone for dual gonorrhea treatment.[7,8,28] Alterations in 23S rRNA are reported to contribute to high-level (MIC ≥256 μg/mL) or low-level (MIC of 4 or 8 μg/mL) azithromycin resistance.[21] In our study, the mutation A2143G (A2059 in *E. coli* numbering), associated with high level resistance was not detected. The mutation C2611T was identified in all 4 alleles of the 23S rRNA gene in all isolates with MIC ≥4 μg/mL, and 1 isolate with MIC of 1 μg/mL contained this mutation in 2 copies of the 23S rRNA gene. Mutation in the *mtrR* gene or its promoter region, which have been shown to increase azithromycin MICs by causing overexpression of MtrCDE pump efflux,[29] was observed in all isolates with decreased susceptibility to azithromycin. Accordingly, *N. gonorrhoeae* isolates with azithromycin MIC of 1 μg/mL were mainly associated with mutations in *mtrR* and its promotor, as previously described.[23,29] Recently, it has been shown that mosaic-like *mtr* loci are responsible for increased azithromycin MIC in *N. gonorrhoeae* (1–4 μg/mL).[24,25] Although 2 isolates in our set had mosaic-like mtr loci, one showed decreased azithromycin susceptibility (1 μg/mL), the second one was susceptible. These loci shared low sequence identity (95%) and further analysis might provide new insights in the role of this locus in *N. gonorrhoeae* azithromycin resistance. Another worrisome finding in our study is that decreased azithromycin susceptibility was associated with isolates harboring *penA* mosaic allele 34. Continued evolution and widespread transmission of these isolates might challenge the effectiveness of current therapeutic options.

In the current study, the molecular typing grouped the isolates from both regions mainly into cluster I, which revealed that ST1407 and very closely related subtypes (e.g., ST10451, commonly found in Ontario) were predominant among *N. gonorrhoeae* isolates with decreased susceptibility to ESC from Argentina and Ontario. ST1407 has been previously shown to be associated with decreased susceptibility to ESC, and responsible for treatment failures to ESC and azithromycin in many countries.[13] This clone is thought to be originated in Japan and spread worldwide, with the first report of ST1407 clone in the United States in 2008.[30] In this study, the molecular characteristics of ST1407 showed similarity to the predominant clone reported in other settings, indicating that importation and further spread within the 2 regions have occurred. The analysis of a larger number of isolates from different areas and different period in time would enable a better understanding of the phylogeny of gonococcal isolates and would more thoroughly

explain the differences between Argentinean and Ontarian isolates. Recent studies from Canada and Argentina showed the ST1407 clone circulating in both countries before 2005 and 2011, respectively.[2,30] Increased awareness of the dissemination of this type of gonococcal strains is crucial. Epidemiological and molecular data on *N. gonorrhoeae* isolates with decreased susceptibility to ESC from Canada and United States are available, but in Latin America are scarce. Therefore, molecular studies are necessary for better understanding of the resistance mechanism and epidemiology in *N. gonorrhoeae* in order to maximize the effectiveness of currently available antibiotics for the treatment of gonorrhea.

In conclusion, international collaboration based on WGS analysis was used to compare contemporary *N. gonorrhoeae* isolates with decreased susceptibility to ESC from Ontario, Canada, and Argentina. Canadian isolates were genetically more heterogeneous, but the international spread of the MDR ST1407 or closely related clones were observed as predominant in both regions. This evidence suggests the need for intensification of surveillance programs and establishment of collaborative projects in regional studies to provide national/regional information in order to establish action plans and public health policies to mitigate emergence and spread of resistant *N. gonorrhoeae*.

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